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Received, November 8, 2001.
Accepted, May 29, 2002.

TRANSPLANTED NEURAL STEM CELLS SURVIVE, DIFFERENTIATE, AND IMPROVE NEUROLOGICAL MOTOR FUNCTION AFTER EXPERIMENTAL TRAUMATIC BRAIN INJURY

OBJECTIVE: Using the neural stem cell (NSC) clone C17.2, we evaluated the ability of transplanted murine NSCs to attenuate cognitive and neurological motor deficits after traumatic brain injury.

METHODS: Nonimmunosuppressed C57BL/6 mice (n = 65) were anesthetized and subjected to lateral controlled cortical impact brain injury (n = 52) or surgery without injury (sham operation group, n = 13). At 3 days postinjury, all brain-injured animals were reanesthetized and randomized to receive stereotactic injection of NSCs or control cells (human embryonic kidney cells) into the cortex-hippocampus interface in either the ipsilateral or the contralateral hemisphere. One group of animals (n = 7) was killed at either 1 or 3 weeks postinjury to assess NSC survival in the acute posttraumatic period. Motor function was evaluated at weekly intervals for 12 weeks in the remaining animals, and cognitive (i.e., learning) deficits were assessed at 3 and 12 weeks after transplantation.

RESULTS: Brain-injured animals that received either ipsilateral or contralateral NSC transplants showed significantly improved motor function in selected tests as compared with human embryonic kidney cell-transplanted animals during the 12-week observation period. Cognitive dysfunction was unaffected by transplantation at either 3 or 12 weeks postinjury. Histological analyses showed that NSCs survive for as long as 13 weeks after transplantation and were detected in the hippocampus and/or cortical areas adjacent to the injury cavity. At 13 weeks, the NSCs transplanted ipsilateral to the impact site expressed neuronal (NeuN) or astrocytic (glial fibrillary acidic protein) markers but not markers of oligodendrocytes (2'3'cyclic nucleotide 3'-phosphodiesterase), whereas the contralaterally transplanted NSCs expressed neuronal but not glial markers (double-labeled immunofluorescence and confocal microscopy).

CONCLUSION: These data suggest that transplanted NSCs can survive in the traumatically injured brain, differentiate into neurons and/or glia, and attenuate motor dysfunction after traumatic brain injury.

KEY WORDS: Behavior, Cell engraftment, C17.2, Head injury, Transplantation

Neurosurgery 51:1043-1054, 2002 DOI: 10.1227/01.NEU.0000027826.68845.CF www.neurosurgery-online.com

Previous studies in experimental traumatic brain injury (TBI) models using fetal central nervous system (CNS) tissue have demonstrated extended graft survival, neurite outgrowth, attenuated neural cell death, and reduced posttraumatic behavioral deficits (37, 44, 45). However, the limited availability of fetal CNS tissue, difficulties in standardization, lack of tissue homogeneity, and potential ethical issues have led to an extensive search for alternative tissue sources for neural transplantation. The isolation and characterization of neural progenitor or stem cells have led to new possibilities for the use of neural stem cells (NSCs) and/or precursor cells as a source for CNS transplantation (13,

18, 24, 49). It has been suggested that neural precursor cell transplantation could have the potential to attenuate anatomic or functional deficits associated with injury or disease in the CNS via cell replacement, the release of specific neurotransmitters, and/or the production of factors that promote neuronal growth (4, 15, 21).

The C17.2 NSCs were derived from a clonal, multipotent mouse progenitor cell line (34). These cells have the ability to self-renew, differentiate into cells of a broad range of neuronal and glial lineages, and populate the developing or degenerating CNS (32). For example, after transplantation into normal newborn mouse cerebellum, these NSCs participate in normal cerebellar development, engraft in an architecturally appropriate, nontumorigenic manner, and differentiate into multiple cerebellar cell types, similarly to endogenous progenitors (42). When engrafted into the cerebellum of the meander mouse, they compensate for and replace the deficient neuronal cell type (32). When transplanted into regions of adult mouse neocortex after targeted photolytic cell death of pyramidal neurons in Layers II and/or III, these C17.2 NSCs integrated into the regions of selective neuronal death and differentiated into neurons resembling those of Layers II and/or III (43). The C17.2 cells are capable of differentiating into various neurotransmitter phenotypes, including dopaminergic (48) and cholinergic neurons (9). Furthermore, when transplanted into the dysmyelinated newborn *shiverer* mouse, the cells showed widespread engraftment and seemed to differentiate into oligodendrocytes with the repletion of deficient myelin basic protein (50). When genetically modified to secrete neurotrophin-3, this clone of NSCs was also shown to survive for up to 2 months, migrate, and differentiate into cells with neuronal and glial phenotypes after transplantation into the rat spinal cord (19). Taken together, these studies suggest that NSCs could potentially be used for the treatment of insults to the CNS to provide widespread replacement not only of therapeutic molecules, such as trophic factors, but also of neural cells.

An estimated 2 million people sustain TBI in the United States each year, and TBI remains the leading cause of long-term neurological disability among children and young adults (29). To date, no clinical therapy has been proven effective in reversing the persistent, devastating neurobehavioral deficits associated with TBI. In the present study, we evaluated the effects of postinjury transplantation of C17.2 NSCs on neurological motor function, cognitive function, and NSC survival and differentiation in the injured brain during a 3-month observation period in a clinically relevant model of experimental TBI in mice.

MATERIALS AND METHODS

Cell Culture and Preparation for Transplantation

NSCs (derived from and modeled by the C17.2 clonal NSC line) were maintained in culture as previously described (32, 42). Before transplantation, the C17.2 and the human embryonic kidney (HEK) cells were trypsinized, gently triturated in serum containing medium to inactivate the trypsin, and washed three times by gently pelleting the cells at low-speed centrifugation (900 $\times$ *g*). The cells were resuspended in phosphate-buffered saline (PBS; pH 7.4) to yield a final concentration of 3 to 4 $\times$ 10^4 cells/μl. Trypan blue was used to assess their viability. The suspension was kept on ice and gently triturated before each injection to keep the suspension dispersed and free of cell clumps.

Animals

Eight-week-old male mice (C57BL/6; mean weight, 24 $\pm$ 1 g) were housed four to six in a cage. The mice were maintained at a constant temperature in a 12-hour light-dark cycle, with lights on at 6:00 AM and access to food and water ad libitum. Brain injury, transplantation, and neurobehavioral testing were performed during the light-on cycle, and the animals were returned to their home cages.

Brain Injury and Transplantation

The Institutional Animal Care and Use Committee at the University of Pennsylvania approved all animal procedures in accordance with federal guidelines (28). The animals (n = 65) were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (65 mg/kg) and placed in a stereotactic headholder (Kopf, Inc., Tujunga, CA). After the skull was exposed, a 5-mm craniectomy was performed over the left parietotemporal cortex between lambda and bregma, with the dura mater left intact. Controlled cortical impact (CCI) brain injury (n = 52) was induced with the use of a pneumatic impactor as described previously (35, 39). Sham-injured animals (n = 13) received anesthesia and all surgical procedures without being subjected to CCI brain injury.

This model of brain injury, developed by Dixon et al. (8) for the rat and modified by Smith et al. (39) for mice, uses a 3-mm rigid impounder driven by a pneumatic piston that is mounted at an angle of 20 degrees from the vertical plane and applied perpendicularly to the exposed dura mater over the left parietotemporal cortex between bregma and lambda at an established velocity and depth of deformation. To obtain the zero point, the impactor tip was lowered until it touched the intact dura mater. The impactor was programmed to deliver the impact at a velocity of 4.8 to 5 m/s and to create a depth of deformation of 1 mm. At the end of the procedure, the craniotomy was covered with a cranioplasty and the scalp was sutured. During surgery and recovery, the mice were placed over a heating pad and maintained at a temperature of 37°C. We previously showed that the temperature of the temporalis muscle in mice remains at 37.6°C during the surgical procedure (27). To minimize variability, the same investigator produced all injuries in the mice.

Brain-injured animals were randomly assigned to one of two groups for transplantation. One group received transplants in the *ipsilateral* hemisphere near the gray matter-white matter interface between the cortex and the hippocampus:

anteroposterior, −2.0 mm bregma; mediolateral, 2.0 mm; dorsoventral, 1.1 mm (C17.2, n = 13; HEK, n = 9). This transplant paradigm was based on previous work in experimental TBI models in which the transplantation of postmitotic human neurons exhibited extended graft survival and viability (31). A second group received transplants in the equivalent location in the *contralateral* hemisphere at the gray matter-white matter interface: anteroposterior, −2.0 mm bregma; mediolateral, −2.0 mm; dorsoventral, 1.1 mm (C17.2, n = 9; HEK, n = 5). Alternatively, a third group received transplants in the contralateral cortex using coordinates that were more caudal to bregma: anteroposterior, −3.0 mm; mediolateral, −1.0 mm; dorsoventral, 1.0 mm (C17.2, n = 5; HEK, n = 4). The contralateral transplantation paradigm was based on previous experiments that demonstrated NSC migration after focal hypoxic-ischemic injury (30) and in models of intraparenchymal brain tumors (1). Additional animals (n = 7) were subjected to CCI brain injury followed 3 days later by NSC transplantation in the ipsilateral hemisphere as described above. These animals were used for histological analysis at 1 week (n = 3) and 3 weeks (n = 4) posttransplantation and were not evaluated for behavioral deficits. *Table 1* depicts the experimental groups used.

Three days postinjury, the animals were reanesthetized with an injection of sodium pentobarbital (65 mg/kg i.p.) and placed into a stereotactic headholder. For transplantation, the cranioplasty was removed for the animals that had received transplants in the ipsilateral (i.e., injured) left hemisphere, and the animals that had received transplants in the contralateral (i.e., uninjured) right hemisphere received a 1-mm diameter burr hole in the skull over the right parietal cortex without the dura being penetrated. Thereafter, each animal received one stereotactic injection of 3 to 4×10^4 cells/μl of either NSCs or HEK cells (1.5 μl using a 26-gauge Hamilton syringe during a 15-minute injection period).

Assessment of Neurological Motor Function

Neurological motor function was evaluated at weekly intervals for 12 weeks after brain injury and transplantation. Evaluation of motor function was performed by blinded, trained observers who used standardized, well-established tests of balance, vestibulomotor function, and coordination (rotarod and rotating pole tests).

Rotating Pole Test

The rotating pole task is a test of coordination and integration of movement, which has been used successfully in previous studies in which experimental models of ischemia and TBI were used (14, 22). The rotating pole test was performed beginning 5 weeks after transplantation and was conducted weekly thereafter until Week 12 postinjury. This evaluation was delayed until 5 weeks on the basis of previous findings that showed that animals that received fetal cortical grafts after middle cerebral artery occlusion needed at least 6 weeks to show improvement in their skills in traversing a rotating pole (14). All animals were required to traverse a 12-mm diameter wooden pole, which is rotated with a constant velocity toward the animal's right side. The animals were acclimated before the first evaluation. Thereafter, they were trained to walk across the wooden pole while it rotated at 5 rpm. After brain injury or sham injury, the animals' skills were assessed by recording the number of foot faults (i.e., slips, jumps) over a marked distance of 50 cm in each of three trials. The average number of foot faults for the three trials was then calculated.

Rotarod Test

The results of the rotarod test, which is a reliable indicator of balance and vestibulomotor function after experimental TBI in rats and mice (12, 35), were compiled by recording the latency of the animal to remain on a linearly accelerating, computer-driven, rotating rod. The rod (36-mm outer diameter) had a rubber surface and rotated with an initial velocity of 5 rpm and an acceleration of 0.5 rpm/s. The trial was terminated if the animal fell off the rod completely or gripped the device and spun around. Two trials were performed at intervals of 5 minutes each, and the latencies (in seconds) of both trials were averaged.

TABLE 1. Description of experimental groups[a]

Group	Transplant	1-week histology	3-week histology	12-week histology	12-week behavior
Sham operation	No transplant	—	—	13	13
Ipsilateral transplant	C17-2 cells	3	4	11	13
	HEK cells	—	—	9	9
Contralateral transplant	C17-2 cells	—	—	12	14
	HEK cells	—	—	9	9

[a] —, not applicable.

Assessment of Cognitive Function

Spatial learning ability was evaluated with the use of the Morris water maze (25) as described previously in brain-injured mice (35). The maze consists of a circular pool 1 m in diameter and painted white inside and filled with water made opaque using nontoxic tempera paint. Mice were trained to locate a hidden stationary platform submerged 0.5 cm below the surface of the water with the use of external cues. The essential feature of the maze is that the animals can escape from the 16 to 18°C water onto the hidden platform after being placed randomly at one of four sites in the pool. In this study, the ability to learn the location of the platform (i.e., latency to reach the platform) over 20 trials conducted during the course of 2 days (10 trials/d) (maximum of 60 seconds for each trial) was assessed at 3 weeks after transplantation. At 12 weeks after transplantation, the location of the platform was changed, and the animals were trained to learn the new location over 20 trials in 2 days.

Histochemical Evaluation of Transplanted NSCs

To determine the survival, localization, extent of engraftment, and differentiation of NSCs in the traumatically injured brain, the mice that were used for behavioral assessment were killed 13 weeks after transplantation with an overdose of sodium pentobarbital (200 mg/kg, i.p.). Transplanted NSCs express *lacZ* gene-encoding β-galactosidase (β-gal), which is detectable with the use of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (42). For X-Gal histochemistry, animals were transcardially perfused with 10 ml cold PBS containing 2 mmol/L $MgCl_2$ and 2 mmol/L ethyleneglycol tetra-acetic acid, followed by 30 ml cold 2% paraformaldehyde in 0.1 mol/L piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) buffer containing 2 mmol/L $MgCl_2$ and 2 mmol/L ethyleneglycol tetra-acetic acid. The brains were removed and postfixed for 30 minutes at 4°C, cryoprotected in 30% sucrose in PBS containing 2 mmol/L $MgCl_2$ overnight at 4°C, embedded in OCT optimal cutting temperature gel (Sakura Finetek, Inc., Torrance, CA), and stored at −80°C. Coronal sections (20 μm) of the entire brain were cut on a cryostat and mounted on gelatinized slides. We evaluated sections from +3.0 to −4.0 mm from bregma randomly selected from the region around the site of transplantation and incubated them with X-Gal (containing 2 mmol/L $MgCl_2$, 35 mmol/L potassium ferricyanide, and 35 mmol/L potassium ferrocyanide in PBS) at 37°C for 14 to 18 hours to generate a blue precipitate in *lacZ*-expressing cells.

Identification of Transplant Phenotype

For immunofluorescence, a polyclonal antibody raised in the rabbit against β-gal (1:500; Cappel Laboratories, Aurora, OH) was used to perform double-labeling of NSCs with cell type-specific markers (40). Briefly, randomly chosen slides from animals with X-gal$^+$ staining were blocked in 5% normal goat serum and then incubated with anti-β-gal antibody overnight at 4°C. The sections were then incubated at room temperature for 1 hour in biotin-SP-conjugated goat antirabbit immunoglobulin G (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The tissue was rinsed and incubated overnight at 4°C with monoclonal antibodies against 1) glial fibrillary acidic protein (GFAP, 1:400; Sigma Chemical Co., St. Louis, MO) to detect astrocytes, 2) 2'3'cyclic nucleotide 3'-phosphodiesterase (CNPase, 1:400; Chemicon International, Temecula, CA) to detect oligodendrocytes, or 3) neuronal nuclear protein (NeuN, 1:1000; Chemicon International) to detect neurons. Diluent without primary antibodies served as a negative control. The next day tissue was rinsed and blocked, as described above, and was incubated for 1 hour at room temperature with Alexa 488-conjugated antimouse immunoglobulin G (1:500; Molecular Probes, Inc., Eugene, OR) and Alexa 594-conjugated streptavidin (1:500; Molecular Probes). The slides were examined using BioRad Radiance 2000 (BioRad, Hercules, CA) with the Nikon TE-300 inverted microscope (Nikon Co., Natick, MA) optimized for two-photon imaging. Images were recorded using BioRad's LaserSharp 2000 NT. Postprocessing of images was performed with Confocal Assistant (BioRad).

Statistical Analysis

To determine whether brain injury produced a significant deficit in motor function or cognition, data from all brain-injured animals (HEK- and NSC-transplanted animals) were combined and compared with sham-operated animals with the use of 1) a one-way analysis of variance (ANOVA) for repeated measurements (time) for the rotarod and rotating pole tests and 2) a *t* test for learning in the Morris water maze. The effect of transplantation in brain-injured animals was analyzed with the use of a two-way ANOVA (cell type × hemisphere of transplantation) for repeated measurements (time). When significant interactions were detected in any ANOVA paradigm, post hoc Student-Newman-Keuls *t* tests were used to demonstrate effects between individual groups. $P < 0.05$ was considered statistically significant.

RESULTS

Neurological Motor Function

Rotating Pole Test

Brain-injured animals had a significantly greater number of foot faults when crossing the rotating pole than did sham-injured animals (injury effect: $P < 0.001$, $F = 48.42$; *Fig. 1A*). Beginning with Week 5 postinjury, brain-injured animals that had received NSCs, regardless of transplantation site, demonstrated fewer foot faults than did brain-injured animals that had received HEK cell transplants (cell effect: $P < 0.001$, $F = 34.87$; *Fig. 1B*). Although the brain-injured animals that had received HEK cells, regardless of transplantation site, exhibited a greater change in rotating pole performance (cell × time: $P < 0.001$, $F = 4.29$), this outcome may reflect improved function in NSC-transplanted animals by Week 5 (*Fig. 1B*).

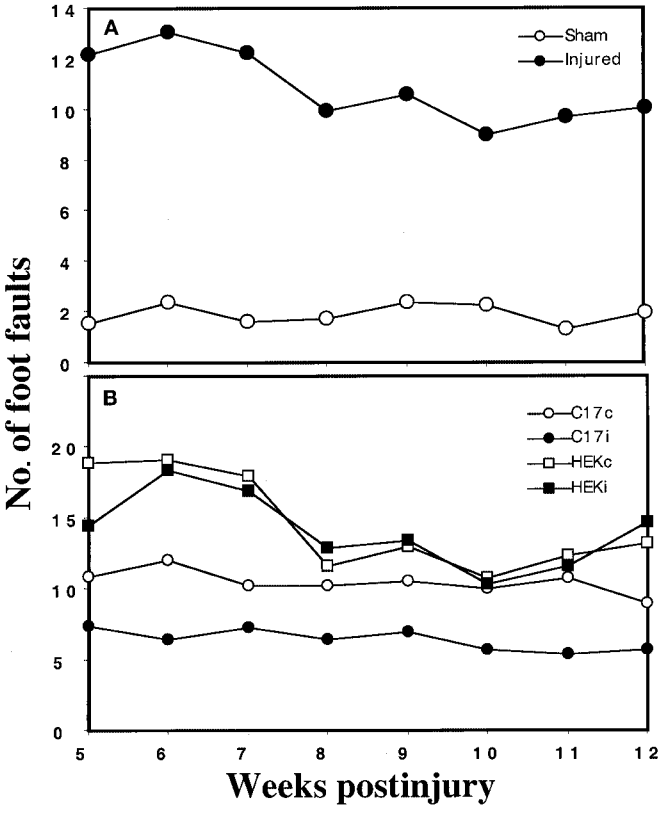

FIGURE 1. *Evaluation of coordination and integration of movement using the rotating pole test.* A, *brain-injured animals (receiving either HEK or C17.2 cell transplants in either hemisphere,* filled circles*) demonstrated a significantly greater number of foot faults when traversing the rotating pole than did sham-operated animals* (open circles). B, *brain-injured animals that received C17.2 cells into either the ipsilateral (*filled circles*) or the contralateral* (open circles*) hemisphere exhibited fewer foot faults compared to brain-injured animals that received HEK cell transplants into the either hemisphere* (squares). Data points *represent average number of foot faults at each time point.*

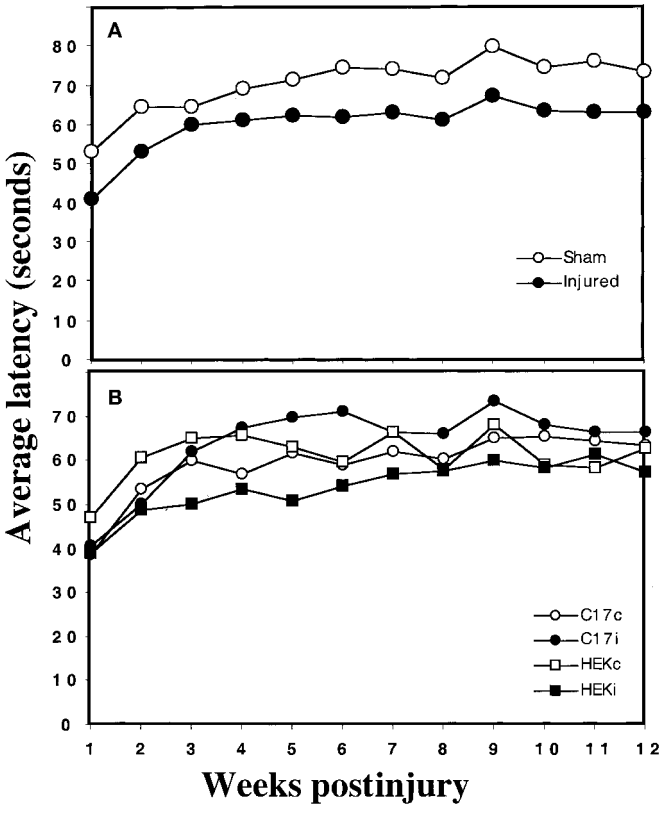

FIGURE 2. *Evaluation of vestibulomotor function and balance assessed using the rotarod test.* A, *brain-injured animals (receiving either HEK cell or NSC transplants in either hemisphere,* filled circles*) demonstrated shorter latencies than sham animals* (open circles*) in the rotarod test.* B, *brain-injured animals that received HEK cell transplants in the ipsilateral hemisphere (*filled squares*) were more impaired than animals that received NSCs in the ipsilateral hemisphere (*filled circles*), and those that received HEK cells in the contralateral hemisphere (*open squares*). Data points represent average latency at each time point.*

Rotarod Test

Brain-injured animals exhibited a highly significant impairment on the rotarod test as compared with sham animals during the 12-week period (injury effect: $P < 0.001$, $F = 19.51$; *Fig. 2A*). Both brain-injured and sham-injured animals demonstrated a significant improvement in performance on the rotarod test during the 12-week period (time effect: $P < 0.001$, $F = 28.31$), which likely was due to acclimation during the first weeks of testing. Although brain-injured animals that received NSCs exhibited longer latencies on the rotarod than did those that received HEK cells, this effect did not reach statistical significance (cell effect: $P = 0.052$, $F = 4.02$). The two-way repeated-measures ANOVA revealed that the efficacy of transplantation depended on both the cell type and the hemisphere of transplantation (cell type × hemisphere: $P < 0.005$, $F = 8.97$; *Fig. 2B*). Brain-injured animals that received NSC transplants in the ipsilateral hemisphere performed better in the rotarod test as compared with animals that received HEK cells in the ipsilateral hemisphere ($P < 0.01$). Brain-injured animals that received HEK cells in the ipsilateral hemisphere exhibited greater deficits that did those that received HEK cells in the contralateral hemisphere ($P < 0.05$).

Cognitive Function

At 3 weeks posttransplantation, brain-injured animals showed a significant deficit in their spatial learning ability in the Morris water maze as compared with sham-operated animals ($P < 0.001$, $F = 50.22$; *Table 2*). At 12 weeks postinjury, brain-injured animals exhibited a mild learning deficit as compared with sham-injured animals ($P = 0.058$, $F = 3.72$). The transplantation of NSCs into either the ipsilateral or contralateral hemisphere after TBI produced no significant beneficial effect on learning ability at 3 or 12 weeks posttransplantation as compared with the transplantation of HEK cells into brain-injured animals.

TABLE 2. Evaluation of the ability to learn a visuospatial task in a Morris water maze at 3 and 12 weeks after transplantation after traumatic brain injury or sham injury[a]

Week	Sham operation	HEK ipsilateral	HEK contralateral	C17.2 ipsilateral	C17.2 contralateral
3	19.6 ± 1.7	33.5 ± 1.2	39.5 ± 3.2	34.1 ± 1.9	34.8 ± 1.9
12	21.8 ± 1.8	25.7 ± 1.8	29.4 ± 2.9	23.9 ± 1.6	25.4 ± 1.4

[a] Brain-injured animals showed cognitive dysfunction as evidenced by longer latency to find the hidden platform (3 wk, $P < 0.001$; 12 wk, $P = 0.058$). No significant differences were detected between brain-injured animals that received C17.2 transplants and animals that received transplanted HEK cells. Data are presented as mean values for latency to find the hidden platform (in seconds) ± the standard error of the mean.

Transplant Survival, Location, and Fate

TBI produced marked cell loss in the left (sensorimotor) parietal cortex and ipsilateral hippocampus at 13 weeks postinjury (*Fig. 3B*). To detect *lacZ*-expressing donor-derived cells, coronal sections of recipient brains were processed for X-gal histochemical examination. Graft survival was defined by the presence of X-gal reaction product. Seven animals were transplanted with C17.2 cells 3 days after TBI and were killed at 1 and 3 weeks to evaluate acute NSC survival. X-gal$^+$ cells were present in three of three brains at 1 week and in two of four brains at 3 weeks. By 13 weeks posttransplantation, 6 (54%) of 11 animals that had received ipsilateral (peri-injury) NSC transplants exhibited X-gal$^+$ cells located in or around the injured cortex or in the dentate gyrus (*Fig. 3B*). Seven (58%)

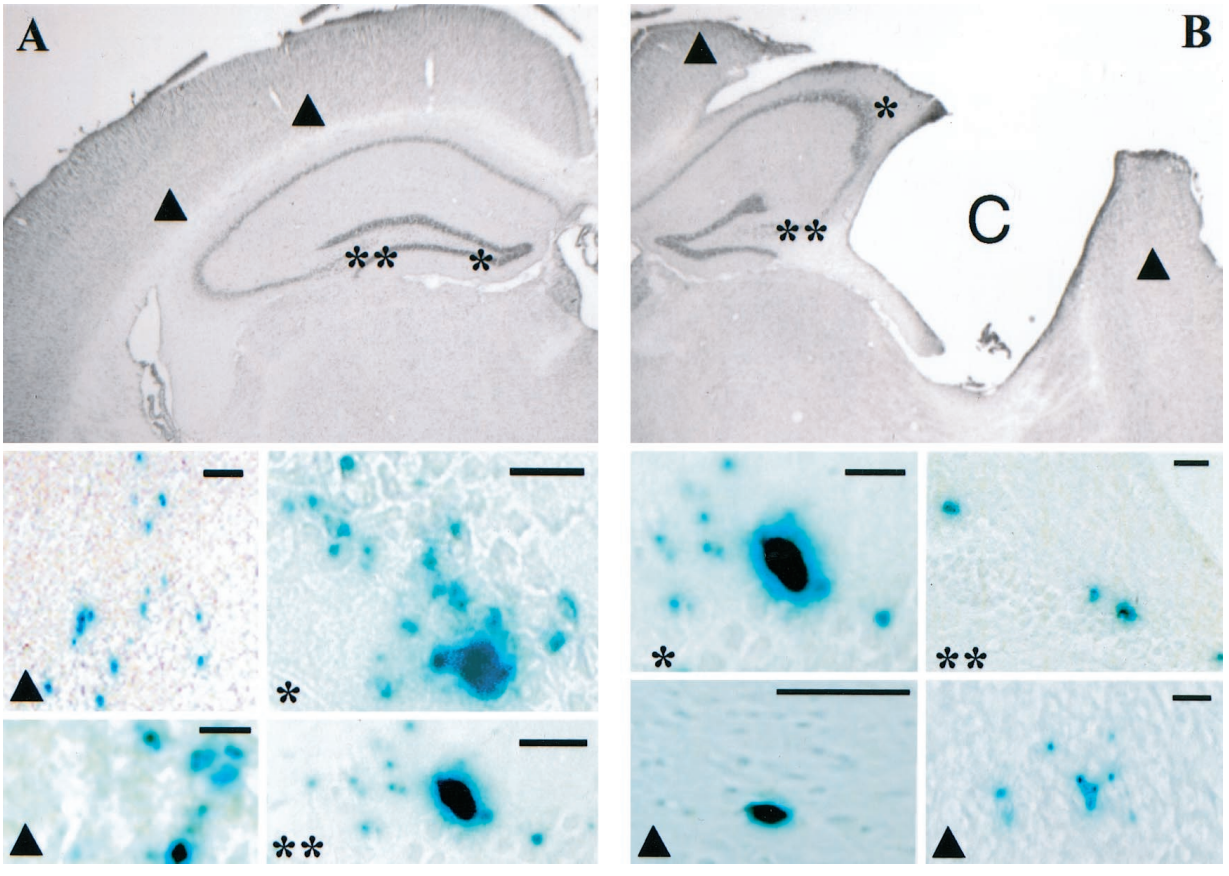

FIGURE 3. *Representative location of X-gal$^+$ C17.2 cells at 13 weeks after transplantation either contralateral (A) or ipsilateral (B) hemisphere. After transplantation into the contralateral hemisphere (A), NSCs were localized in the cortex (▲), dentate gyrus (**) or CA_1 (*) region of the hippocampus. After ipsilateral hemispheric transplantation (B), X-gal$^+$ cells were located in the cortex (▲) surrounding the injury cavity (C), the granule cell layer of the dentate gyrus (**), and the CA_3 region (*) of the hippocampus. Both sections (A, B) depict levels taken at bregma −2 mm.* Scale bars, *1 mm (A, B); 20 μm for all other panels.*

of 12 animals that had received transplants in the contralateral hemisphere exhibited X-gal$^+$ cells in the cortex, the dentate gyrus, or the CA_1 region of the hippocampus (*Fig. 3A*). No migration of C17.2 cells transplanted in the hemisphere contralateral to the injured hemisphere was observed in our study. No differences were observed with regard to behavioral outcome (i.e., cognition or motor function) between animals that exhibited documentable surviving cells and those engrafted animals in which cells could not be localized at 13 weeks posttransplant. In animals that had received ipsilateral NSC transplants, cellular staining for X-gal in both transplantation groups was detected in the lower blade of the hippocampal dentate gyrus and in the cortex lateral to the contusion cavity. At earlier time points (1 and 3 wk), NSCs were detected at the hippocampus-thalamus interface in the cortex surrounding the cavity and in the thalamus (*Fig. 4, A* and *B*).

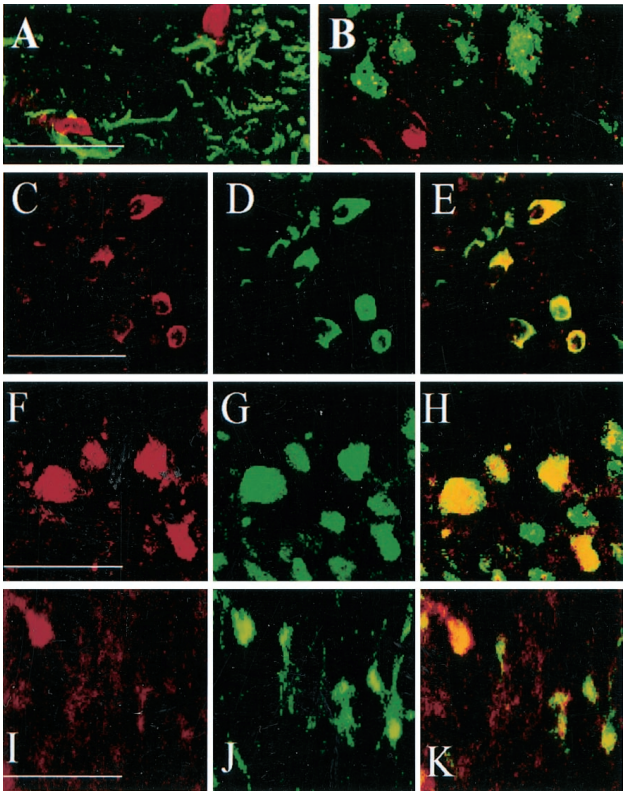

FIGURE 4. *Representative confocal photomicrographs demonstrating the differentiation of transplanted NSCs at 3 weeks* (A, B) *or at 13 weeks* (C–K). *Brain sections were stained first with anti-β-gal antibody* (red, A–C, F, *and* I) *to identify NSCs and then with either anti-NeuN to identify neurons* (green, B, D, *and* G) *or anti-GFAP to identify reactive astrocytes* (green, A *and* J). *At 3 weeks after transplantation, β-gal$^+$ NSCs* (A *and* B) *did not colabel with either anti-GFAP* (A) *or anti-NeuN* (B). *At 13 weeks after transplantation in the ipsilateral hemisphere, β-gal$^+$ NSCs were immunoreactive for NeuN* (yellow cells *in* E) *and GFAP* (yellow cells *in* K). *After transplantation in the contralateral hemisphere, β-gal$^+$ NSCs were immunoreactive for NeuN only* (yellow *cells in* H). Scale bar, *50 μm.*

These cells did not seem to have differentiated, because they did not label with neuronal (NeuN) or astrocytic (GFAP) markers. No X-gal$^+$ cells were detected in sham-operated (i.e., uninjured) animals or in animals that had received HEK cell transplants.

To identify whether NSCs that survived to 13 weeks posttransplantation had differentiated into neurons or glia, tissue sections were immunolabeled for β-gal- and cell-specific markers. In animals in which X-gal$^+$ NSCs were engrafted into the ipsilateral hemisphere, 60% of cells were double-labeled for X-gal together with the neuronal marker NeuN (*Fig. 4, C–E*), whereas 40% exhibited double-labeling with an astrocytic marker (GFAP) (*Fig. 4, I–K*), suggesting that transplanted NSCs had differentiated into either neurons or astrocytes within the traumatically injured mouse brain. No double-labeling for the oligodendrocytic marker (CNPase) was observed in this group. In animals that had received contralateral NSC transplantation, NSCs double-labeled for X-gal and neuronal (NeuN) markers only (*Fig. 4F–H*) and did not co-localize with GFAP or CNPase.

DISCUSSION

The results of this study demonstrate that C17.2 NSCs transplanted intracerebrally in the acute period after TBI survive, differentiate, and attenuate posttraumatic neurological deficits in the chronic postinjury period. Brain-injured mice that received NSC transplants showed significantly improved performance in the rotating pole test during the 8-week observation period. In addition, brain-injured animals that received NSCs in the ipsilateral hemisphere exhibited improved performance in the rotarod test during the 12-week observation period. Vehicle-treated, brain-injured animals were intentionally not incorporated into our study design, because we were interested in the effect of an intracerebral intervention and because a non-neuronal cell line (HEK cells) was used to control for mass effects of cellular engraftment. No overt differences were observed with regard to cognitive and motor function between brain-injured animals that received HEK cell transplants in the present study and our historical brain-injured control animals evaluated at 1 and 4 weeks postinjury. We observed no additional evidence of exacerbated inflammatory response in brain-injured animals that received HEK cell transplants, consistent with previous reports demonstrating that transplantation of renal embryonic precursor cells does not elicit an immunogenic response (16). Moreover, all animals were treated daily with cyclosporine specifically to prevent the development of a transplant-induced inflammatory response.

In humans, TBI is associated with disabilities affecting mobility, motor function, and motor coordination (11). To elucidate the effect of NSC transplantation on neurobehavioral outcome after TBI, we systematically evaluated motor deficits using a variety of functional tests, including the rotarod test for vestibulomotor function and balance and the rotating pole test to measure deficits in coordination and integration of

movement. The model of TBI used in this study produced sustained neurological motor dysfunction, as indicated by decreased latency time on the rotarod and a significant increase in the number of foot faults when traversing the rotating pole in brain-injured animals. The magnitude of acute motor dysfunction observed in the present study was consistent with previous studies of lateral CCI brain injury (12, 26, 35).

Our results suggest that either ipsilateral or contralateral NSC transplantation can significantly attenuate specific postinjury motor deficits. A recently published study revealed that the transplantation of embryonic stem cells directly into the site of injury after experimental spinal cord injury also improved locomotor function (23). Studies in models of acute and chronic injury to the CNS have described significant behavioral effects after neural grafting (5, 14, 38), whereas the transplantation of NSCs into the demyelinated *shiverer* mouse brain has been shown to ameliorate phenotypic behavioral symptoms (50). In our study, the transplantation of NSCs into the contralateral hemisphere reduced deficits on the rotating pole, whereas transplantation into the ipsilateral hemisphere improved both rotating pole scores and rotarod latencies. Because both transplantation paradigms led to a significant reduction in neurological deficits on the rotating pole with no additional recovery during the next 7-week period, maximal improvement in performing this task seemed to occur at or before 5 weeks posttransplantation. Cognitive dysfunction (i.e., learning deficits) was observed in brain-injured animals at 3 weeks posttransplantation; however, the transplantation of NSCs had no beneficial effect on postinjury learning ability as compared with the transplantation of HEK cells at 3 or 12 weeks after transplantation. In animals that received transplants, no differences were observed in cognitive or motor function between those that had documented X-gal$^+$ surviving cells at 13 weeks and those in which the transplants could not be localized. Our inability to locate engrafted C17.2 cells at 13 weeks posttransplantation in a subset of animals is likely due to the diminution of X-gal expression in these cells over time, because previous studies in which transduced C17.2 cells were engrafted into injured spinal cord documented significant down-regulation of the β-gal reporter gene by 2 months posttransplantation (17, 19).

Thirteen weeks after their transplantation under the contusion cavity, NSCs were found in the ipsilateral hippocampus and in the cortical parenchyma adjacent to the injury cavity. NSCs transplanted into the contralateral hemisphere were also detected in the contralateral hippocampus and the contralateral cortex. The NSCs in the injured cortex were found to express neuronal and astrocytic markers, implicating differentiation into neurons and glia, whereas the NSCs transplanted into the uninjured contralateral cortex showed an almost exclusively neuronal phenotype. The reasons underlying these apparent variations in differentiation remain unclear, although the demand for both neurons and glial cell replacement may be far greater in the areas of the injured hemisphere that have sustained significant tissue loss. Previous histopathological analysis after CCI brain injury revealed substantial cortical tissue loss in the region of impact and selective hippocampal neuronal cell loss (39). The differentiation of the NSCs in the ipsilateral hippocampal areas may be related to the extensive cell loss observed in these areas after CCI brain injury, because previous studies showed that NSCs possess the ability to migrate into degenerating CNS areas (34, 40, 42). The discovery of differentiated and undifferentiated transplanted NSCs in hippocampal and/or cortical areas may be due either to variability in injection sites between animals or, more likely, to stem cell migration. The mechanisms underlying the hippocampal accumulation of NSCs after contralateral cortical transplantation are also not completely understood. It was previously reported that the proliferation of neuronal precursor cells in the dentate gyrus is accelerated after transient forebrain ischemia in mice (46). Neurogenesis has been found to occur in the adult hippocampus in humans and rodents (7, 10, 33), and multipotent stem and/or progenitor cells have been isolated from this brain area (18, 33). Therefore, specific hippocampal regions may provide a favorable milieu for transplanted NSCs.

The mechanisms underlying the beneficial effects of NSCs on functional motor outcome in this study may be related in part to cell replacement, a reduction in tissue loss or in glial scarring, or the potential neurotrophic properties of the C17.2 cell. We observed that transplanted NSCs had the capability of differentiating into both neurons and glial cell types. This pattern of differentiation was previously shown in a study in which it was reported that NSCs have the ability to differentiate and to replace dysfunctional apoptotic neuronal cells (43). Recently, Teng et al. (47) suggested that the efficacy of C17.2 cells in mediating functional recovery after traumatic spinal cord injury might also be due to a reduction in tissue loss from secondary injury processes as well as from diminished glial scarring. Other potential neuroprotective mechanisms underlying the beneficial behavioral effects of NSC transplants may include production and/or secretion of trophic factors, which may lead to neuroprotection and functional restoration, as reported in earlier studies in which fetal transplantation was used as an experimental therapeutic strategy for TBI (37, 38). Recently, Lu et al. (20) demonstrated that C17.2 cells have the ability to produce neurotrophic factors such as brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. The effectiveness of the contralateral C17.2 transplants may be due in part to the neurotrophic properties of this NSC population.

Neural transplantation has been suggested to be potentially useful as a therapeutic intervention in several CNS diseases, including Parkinson's disease (6), Huntington's disease (3), ischemic brain injury (36), and spinal cord injury (23). Our study demonstrates that the transplantation of NSCs into the ipsilateral (injured) or contralateral hemisphere 3 days after TBI promotes the improvement of specific components of motor function in the chronic postinjury period. The advantages of using NSCs rather than fetal tissue or differentiated neurons for CNS transplantation in the traumatically injured

brain include 1) the ability to self-renew, differentiate into cells of neuronal and glial lineages, and populate the developing or degenerating CNS (34, 42); 2) the potential to provide diffusible (e.g., trophic factors) and nondiffusible (e.g., cell-cell contact signals, myelin) factors (41); and 3) the ability to genetically engineer cells to produce factors that might attenuate secondary or delayed disorders and/or damage and provide regeneration after TBI (2, 41). Our data suggest that NSC transplantation may potentially be a useful therapy to improve neurological outcome after injury to the CNS. Further studies of this novel neuroprotective strategy are warranted.

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Acknowledgments

We thank Dr. Mary Putt for statistical support and Dr. James F. Sanzo for assistance in confocal microscopy procedures. We also thank Jeanne Marks for her assistance in preparing this manuscript. We gratefully acknowledge the participation of Dr. Yang D. Teng of the Snyder laboratory in the maintenance, preparation, and transplantation of the neural stem cells used in these experiments. Dr. Teng is now affiliated with the Department of Neurosurgery, Children's Hospital and Harvard Medical School. This work was supported by a Merit Review grant from the Veterans Administration (TKM); a grant from Layton Bioscience (TKM); National Institutes of Health Grants NINDS RO1-NS-40978 (TKM), P01-NS08803 (TKM), NIGMS RO1-GM34690 (TKM), NINDS RO1-NS33852 (EYS), NINDS RO1 NS34247 (EYS); and a March of Dimes grant (EYS).

COMMENTS

In this study, the authors evaluated the effects of the transplantation of neural stem cells (NSCs) in a murine cortical injury model. After sustaining cortical injuries, the animals demonstrated clear deficits in motor and learning functions. These deficits were not reversed after the transplantation of embryonic kidney cells or NSCs into the contralateral hemisphere. NSC transplantation into the ipsilateral damaged hemisphere, however, resulted in clear improvement in motor function but no change in the cognitive test used. The authors demonstrate that the NSCs differentiated into neuronal cells, which were marked by NeuN in both the ipsilateral and contralateral hemispheres. The ipsilateral hemisphere also contained NSC-derived glial cells.

This article is important because of the implication that neuronal transplantation using NSCs might be of benefit in ameliorating neurological deficits after traumatic brain injuries (TBI) in humans. The real question is whether such studies are ready to be implemented in clinical trials. The long-term effects of these transplants are unknown. The current study lasted for only 3 months. Furthermore, it seems logical that similar studies should be done in primates before clinical testing can be justified. Such a study obviously would be much more complex and expensive than a murine study. Whether the experience with human transplantation in patients with Parkinson's disease is adequate to determine the safety of all NSC transplantation is unclear.

The authors make an important point that the mechanism of the observed effects is unknown. The question is, are the transplanted and differentiated cells incorporated into the neural circuitry, thereby resulting in improved motor effects? It is more likely that some trophic factors produced by the NSCs allow better repair or less long-term cell death. These questions are crucial, because if the latter mechanism is operant, then other less complex methods of neural functional enhancement will be available. There is little doubt that understanding the details of NSC biology and transplantation will be the basis for future treatments of a wide variety of neurological problems, including those related to trauma.

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The authors have studied the ability of transplanted murine NSCs to alter cognitive and motor deficits after TBI. They show that motor deficits but not cognitive dysfunction can be improved after TBI in this model. Interestingly, NSCs survive up to 13 weeks after transplantation. That NSCs transplanted ipsilateral to the impact site express different markers from those transplanted contralaterally is also of note. Why motor recovery but not cognitive recovery occurred early is an interesting question that needs to be examined in future studies.

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NSCs are the salvation of the brain! Regardless of whether the neurological insult is the result of a degenerative process, trauma, or stroke, NSCs are the answer. Of course, neurosurgeons still do not know how to ask the proper questions. We do, however, know how to obtain NSCs. These cells can easily be obtained from committed precursors in embryos (10), from in vitro expanded mesencephalic progenitors (6, 9),

from immortalized NSCs (12), from embryonic stem cells (5), and even from the dead (8). The problem is which stem cells to use, how to use them, and how to control them, none of which is currently known. The major problem with NSCs is their rapid loss in number and their migration throughout the brain. There is also the potential for inappropriate neural connections' resulting in adverse behavior. The transplant of partially differentiated embryonic stem cells in the Parkinson's model demonstrated that only 56% of the animals had surviving grafts that contained the appropriate dopaminergic neurons, whereas 20% had lethal teratomas and 24% had no cells survive at all (1).

This article is not different in that the number of surviving cells undoubtedly is remarkably small compared with what the number that were transplanted. Migration undoubtedly occurred to a degree, which is not really clarified in this study. It is stated that there was no migration to contralateral side, yet the early periods when this might have occurred were not evaluated. Furthermore, only 7 of 12 of the contralateral injections had cells that could still be identified in the 12-week follow-up period. Indeed, no differentiation could be observed during the first 3 weeks after transplantation. Fortunately, the C17.2 progenitor NSCs in this study did not demonstrate any tendency toward teratoma. Some of these cells did in fact become neurons and astrocytes but not, apparently, oligodendroglia cells. This finding was discovered, of course, with very limited antibody profiling, and, despite problems with the loss of expression of 5-bromo-4-chloro-3-indolyl β-D-galactoside, greater immunological differentiation could have been examined. I wonder why nestin was not used to evaluate many of these nondifferentiated cells. Further characterization of the cellular immunocytochemistry is certainly necessary to determine the exact nature of the surviving cells.

Blood can be turned into brain in vitro (2), and brain can be turned into blood (7), but the desired behavioral responses are still difficult to obtain in vivo (3). Behavioral improvement was observed in the rotating pole test and the rotarod test after ipsilateral injections and only in the rotating pole test after contralateral injections, but no improvement in cognition was observed in either group. Further controls and more sophisticated behavioral testing are obviously required. A key factor to be determined is whether cells are required or whether there is simply a neurotrophic effect or other generalized improvement independent of the need for cellular therapy. That no differences were observed in motor behavior between animals with and without surviving grafts is disconcerting. Even more important is the need for long-term evaluation, which in some instances demonstrates deterioration of initial improvement in a traumatic model such as this one (RAE Bakay, unpublished data).

I encourage the authors to conduct additional studies. It is far too soon to anticipate, after a few rat studies, that NSCs are ready to be studied in clinical trials. Concerns have been raised about host and graft cell fusion (11, 13) as well as about the transdifferentiation of NSCs transplanted for one purpose (hematopoietic) and ending up in other tissues (ectoderm and endoderm) (4). Far too little is known and far too much potential danger exists to conduct extensive studies and to characterize behavior in the best animal models available. In most cases, this also means conducting studies in the monkey to allow at least some degree of understanding of the problems in sizing upward. The problems inherent in studying humans first and acquiring animal data later are well emphasized by the adrenal medullary transplantation fiasco. Many of the problems that exist for fetal tissue are still true for stem cells, regardless of origin. Stem cell technology promises unlimited supplies of sterile, high-quality cells for implantation that can be produced with good manufacturing practices. They still require extensive study and evaluation to provide safe and efficacious treatment in the future.

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The most exciting prospect for the future treatment of neurological diseases is the potential to restore function lost as a result of natural or pathological loss of cellular and struc-

tural elements. The authors present an interesting study that provides evidence that an NSC clone can attenuate neurological motor deficits in a murine model of TBI.

Although animal behavior models contain inherent difficulties, the authors demonstrate a statistically significant reduction in the number of foot faults in the rotating pole test, a well-characterized test of coordination and integration of movement. No significant improvements were detected in either the rotarod test of balance and vestibulomotor function or the Morris water maze test of cognitive function.

Some questions remain, however. For example, to what extent transplanted NSCs survive is not clear. At 13 weeks, 6 of 11 of the animals that underwent ipsilateral transplantation demonstrated identifiable surviving NSCs on the same order as did the 7 of 12 animals that underwent contralateral transplantation. The authors attribute this finding to the down-regulation of the β-galactosidase reporter gene over time. Of the four animals that were killed 3 weeks after transplantation, however, only two of the animals demonstrated detectable NSCs. Furthermore, there was no observed difference in the outcomes for the animals that exhibited documentable surviving cells from the outcomes in animals in which NSCs could not be detected at 13 weeks. Therefore, the presence of detectable NSCs does not seem to be a predictor of outcome in this model.

The mechanism underlying the improvement in motor function is also unclear. The authors suggest that the mechanism could be cell replacement, a reduction in tissue loss or glial scarring, or the neurotrophic effects of NSC. These effects could in part explain a measurable improvement because of transiently surviving NSCs. The authors demonstrate a difference between the differentiation and migration fates between the NSCs that were transplanted ipsilaterally and those that were transplanted contralaterally. Again, one can only speculate regarding the underlying beneficial effects of these differences. Whatever the mechanism, however, the data from this study show a beneficial effect of NSCs on motor function after TBI. Furthermore, the immunohistochemical studies show that at least some of the NSCs survive for up to 13 weeks and differentiate into neurons and glia, adding to a growing body of evidence in the literature for this effect.

It is known that in normal central nervous system development, precursor cells are subject to a preprogrammed set of intrinsic signals to determine their fate. However, this process is modulated by temporally and spatially organized extrinsic signals (2). A similar process is thought to exist with NSCs (1, 3). NSCs are thought to exist in a niche that provides the extrinsic signals to modulate its intrinsically determined fate. The elucidation of these intrinsic and extrinsic signals is an extremely active field of investigation. As the scientific community further understands these processes and mechanisms for the beneficial effects of NSCs, an intriguing question arises: are there ways to artificially modulate these signaling processes to maximize the chances that the beneficial effects will occur? It seems that the potential efficacy of cellular transplantation therapy for neurorestoration is critically dependent on the answer to that question.

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Details from Mount Fuji and Shore of Miho, *by Kano Tanyu. 17th century (Edo period); from a pair of six panel screens; ink, colors, and gold on paper. Courtesy, Asian Art Museum of San Francisco, The Avery Brundage Collection.*